litis (*10*). Thus, flurbiprofen, even at a dose that was too small to elicit an anti-inflammatory response (*11*), had a dramatic effect on alveolar bone loss without supplemental local treatment.

The etiological factors in chronic destructive periodontal disease are not well understood. It seems likely from our data that one of the major biochemical pathways of bone resorption in beagle periodontal disease may be mediated by cyclooxygenase. The use of flurbiprofen to further probe pathologic mechanisms and to treat bone resorption disease has promise.

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8. Gingival inflammation about each tooth was scored on a scale of 0 to 3 with the index of Rosenberg *et al.* [*J. Periodontol.* **37**, 208 (1966)]. Inflammation was not significantly reduced in the treatment period from the baseline pretreatment values ($P > 0.05$).
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11. The average blood level of flurbiprofen measured at day 14 of treatment was 2.7×10^{-6} *M*. While this drug is metabolized much more rapidly in man (half-time, 5 hours) than in the dog (half-time, 36 hours) serum equilibrium levels of flurbiprofen in dogs given drug at 0.02 mg/kg once a day ranged from 0.4 to 1 μg/ml. In man, peak levels are 4 to 6 μg/ml. Multiple dosing in humans results in serum levels one order of magnitude higher than in the dog.
12. This research was supported by a grant from the Department of Hypersensitivity Diseases Research, Upjohn Company. We thank Jayne Gandrup, Anne Schmitt, and Quaife Nichols.

18 June 1984; accepted 31 October 1984

Use of Restriction Fragment Length Polymorphisms to Determine the Clonal Origin of Human Tumors

Abstract. *A novel strategy to determine the clonal origin of human tumors has been devised. The strategy involves the use of a cloned polymorphic X-chromosomal gene and two restriction endonucleases. The first endonuclease distinguishes the paternal and maternal copies of the gene through a DNA polymorphism of restriction fragment length. The second endonuclease distinguishes active from inactive copies of this gene through changes in DNA methylation. As illustrations of this strategy, three human cancers were each shown to be monoclonal. The analysis described should have a wide variety of clinical and experimental applications.*

Knowledge of the clonal origin of a tumor has important conceptual and practical implications. A multiclonal tumor is logically inconsistent with the premise that a rare genetic event (for example, DNA mutation or chromosomal rearrangement) was responsible for formation of the tumor. Indeed, the assumption that tumors are monoclonal is a cornerstone of somatic mutation theories of carcinogenesis (*1*). Consistent with such theories, some human cancers, particularly leukemias and lymphomas, have been shown to be monoclonal (*2, 3*). However, several multiclonal human tumors have been reported (*4*). In animals, too, some experimentally induced cancers are polyclonal in origin (*5*). In humans, a major limiting factor in the clonal analysis of tumors is the availability of a technique that can be generally used to assess clonality. The analysis of glucose-6-phosphate dehydrogenase protein variants has yielded unambiguous and valuable information on clonality, but it is only applicable in 1 of 50 human tumors (*2*). We describe here a new strategy, based on the analysis of DNA polymorphisms of restriction fragment length, for determining the clonal origin of human tumors. To illustrate this strategy, we demonstrated that each of three human cancers was monoclonal.

This analysis of clonal origin is based on three underlying principles: (i) Only one X chromosome in each female cell is active (*6*). This activation occurs at an early step in embryogenesis and is stable throughout the lifetime of the cell, even if that cell is cultured in vitro or becomes neoplastic (*6, 7*). (ii) Activation of many genes, including those on the X chromosome, is accompanied by changes in methylation of cytosine (C) residues (*8*). Although these methylation changes do not affect all sites that potentially can be methylated (*8, 9*), they occur consistently at some sites and are easily monitored at those sites by using restriction endonucleases that have the capacity to recognize methylated C residues (*10*). (iii) The maternal and paternal copies of the X chromosome in female cells can be distinguished at the DNA level, using restriction fragment length polymorphisms (RFLP) (*11*). These polymorphisms are normal variations in DNA sequence that occur in the human population.

The strategy thus involves the use of a cloned polymorphic X-chromosomal gene and two restriction endonucleases. The first endonuclease distinguishes the maternal and paternal copies of the gene through an RFLP. The second endonuclease distinguishes active from inactive copies of this gene, through methylation changes. If the tumor developed from one cell—that is, if it was monoclonal—the paternal copy of the gene will be cleaved by the second enzyme in a much different fashion from the maternal copy, since the paternal copy will be either active in all the cells of the tumor or inactive in all the cells of the tumor. Conversely, in polyclonal tumors or normal tissue, approximately half the cells will have an active paternal gene and half will have an active maternal gene, so that the paternal and maternal copies of the gene will be affected identically by digestion with the second enzyme.

A gene that we have found useful for this technique is the hypoxanthine phosphoribosyltransferase (HPRT) gene. A map of the relevant segment of the gene is shown in Fig. 1A. The Bam HI endonuclease sites B_1 and B_3 are present in all X chromosomes, but 16 percent of X chromosomes contain an additional Bam HI site, B_2 (*12*); hence, 27 percent of females will be heterozygous at this locus. There are at least six Hha I sites within this region of the gene (Fig. 1A) (*13, 14*). Hha I cleaves at the sequence GCGC (G, guanine), but does not cleave this sequence when either C is methylated (*15*). Hha I site 1 is unmethylated in active chromosomes (*13, 14*); cleavage at

site H_1, however, will change the mobility of the Bam HI fragment by less than 3 percent, and this small change in mobility will barely be detected on agarose gels. Sites H_2 to H_6 are each methylated in over 95 percent of active X chromosomes, but at least one of sites H_2 to H_6 is unmethylated in virtually 100 percent of normal inactive X chromosomes (*14, 16*).

On the basis of these facts, a graphic depiction of the results expected to be obtained in monoclonal as opposed to polyclonal tumors is shown in Fig. 1B. Tumor DNA from a heterozygous female is digested with Bam HI, or with Bam HI plus Hha I. DNA samples are then fractionated by electrophoresis in an agarose gel, transferred to nitrocellulose, and hybridized with the probe indicated in Fig. 1A. Digestion with Bam HI alone reveals two restriction fragments of 12 kb and 24 kb (Fig. 1B, lane 1). The intensity of the 24-kb fragment is usually 50 to 80 percent that of the 12-kb fragment. [This is because the size of the DNA that we use (prior to endonuclease digestion) is 50 to 150 kb; a simple calculation shows that randomly generated DNA fragments spanning sites B_1 and B_3 would be represented less often in DNA samples of this size range than would fragments spanning only B_1 and B_2.] If a tumor is monoclonal, with the X chromosome containing the 12-kb fragment active (Fig. 1B, lane 2), the 12-kb fragment will be infrequently cleaved at sites H_2 to H_6 by Hha I, leaving a 12-kb fragment that has an intensity 80 percent or more of that seen in lane 1. In the same digest, the 24-kb fragment will completely disappear after digestion with Hha I (Fig. 1B, lane 2). Conversely, if the tumor is monoclonal with the X chromosome containing the 24-kb fragment active (Fig. 1B, lane 3), the 12-kb fragment will disappear while the intensity of the 24-kb fragment will still be substantial. In a polyclonal tumor (or normal tissue), the result shown in Fig. 1B, lane 4, will be obtained. The 12- and 24-kb bands will both decrease by 50 to 60 percent in intensity compared to lane 1. The pattern in lane 4 is clearly different than those in lanes 2 and 3, thus differentiating between monoclonal and polyclonal tumors.

An important control for the experiment outlined above is the demonstration that the amount of intact 12- or 24-kb Bam HI fragment in a DNA sample is quantitatively reflected in the autoradiograph obtained after gel electrophoresis, transfer, hybridization, and exposure to film. The dilution experiment in Fig. 1C showed that this criterion was met: the amount of 12- or 24-kb Bam HI fragment loaded in a gel lane was linearly related to the autoradiographic intensity of the corresponding band obtained at the end of the experiment over more than a tenfold range. Hence a 10 percent contamination of normal cells in a monoclonal tumor could easily be detected.

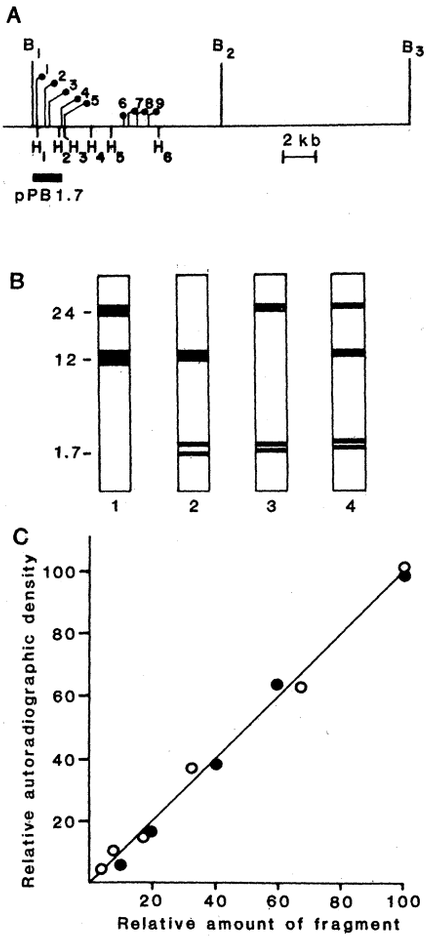

Fig. 1. Restriction map of the 5′ part of the HPRT gene. Abbreviations: B_1 to B_3, Bam HI restriction sites; H_1 to H_6, Hha I sites; 1 to 9, Hpa II sites. The region contained in clone pPB1.7 (*23*) used as hybridization probe is indicated by the 1.7-kb bar. The 5′ end of the messenger RNA is located 250 bp to the left of B_1, and transcription continues through B_3 (*13, 14*). (B) Diagrammatic representation of results that would be expected with monoclonal and polyclonal tumors (see text). Lane 1, Bam HI digest; lanes 2 to 4, Bam HI plus Hha I digests; lane 2, monoclonal tumor, 12-kb allele, active; lane 3, monoclonal tumor, 24-kb allele active; lane 4, polyclonal tumor. The pattern of the low molecular weight bands that appears after Hha I digestion varies between individual DNA samples, depending on the methylation status of sites H_1 to H_6 in the inactive chromosome. At least one of sites H_2 to H_6 is unmethylated in the inactive chromosome. (C) Demonstration of linear relationship between the amount of an HPRT Bam HI fragment in a DNA sample and the resultant intensity of the corresponding band upon autoradiography. Various amounts (0.38 to 7.5 μg) of DNA containing 12- or 24-kb Bam HI fragments were cleaved with Bam HI, subjected to agarose gel electrophoresis, and, after Southern transfer, hybridized to the HPRT probe (see the legend to Fig. 2 for details). The 12-kb (○) and 24-kb (●) fragment bands were scanned with a Clifford Densicomp model 445 densitometer and the intensities determined with a Hewlett-Packard 9874A digitizer using film exposures of 15, 30, and 60 hours. Results were normalized to the intensities obtained with 7.5 μg of DNA.

An example of the autoradiographic results obtained with normal polyclonal tissues is presented in Fig. 2A. Digestion with Hha I of a 24-kb Bam HI fragment from an active chromosome (from a male) resulted in only a slight decrease in its intensity (Fig. 2A, lanes 1). In contrast, a female homozygous for the 24-kb allele showed 50 to 60 percent decrease in intensity of the 24-kb Bam HI fragment upon digestion with Hha I, reflecting the fact that each of these alleles is inactive in approximately one-half of the cells (Fig. 2A, lanes 2). Concomitantly, new fragments of lower molecular weight appeared, corresponding to the expected products of Hha I digestion (see Fig. 1A). In Fig. 2A, lanes 3, an active 12-kb allele was shown to be only slightly affected by Hha I, as expected. In contrast, in normal tissue from a heterozygous female, both the 12-kb and the 24-kb alleles decreased in intensity by 50 to 60 percent after Hha I digestion (Fig. 2A, lanes 4). These patterns were found to be invariant in DNA samples prepared from normal human bone marrow, kidney, peripheral blood lymphocytes, liver, uterine myometrium, and colonic mucosa.

Examples of the patterns seen with cancers from three females who were heterozygous at the Bam HI polymorphic site B_2 are shown in Fig. 2B. In a patient with acute myelocytic leukemia, the neoplastic cells were clearly monoclonal, with the 24-kb allele inactive in all the cells of the tumor (Fig. 2B, lanes 1). In contrast, bone marrow from a normal patient had the expected polyclonal pattern (Fig. 2B, lanes 2). Two solid tumors, a leiomyoma and a Wilms' tumor, were also monoclonal (lanes 3 and 5). In the leiomyoma (lanes 3) the 12-kb allele was inactive, while in the Wilms' tumor (lanes 5) the 24-kb allele was inactive. Faint bands of 12-kb in lane 3 (Bam HI plus Hha I) and 24-kb in lane 5 (Bam HI plus Hha I) could be seen on long exposures of the audioradiograph. This pattern was consistent with the small amount of normal connective tissue found in solid tumors.

This technique thus appears to have the selectivity and sensitivity to determine whether a cell population is mono-

clonal or polyclonal. What are the potentially limiting factors in this analysis? (i) It has been noted that the genome of human cancer cells is sometimes aberrantly methylated (*17*). While we have not seen abnormal hypomethylation of the HPRT gene in human tumors, we have found 4 tumors (of 14 examined) in which sites H_2 to H_6 were resistant to Hha I digestion in both the active and inactive chromosomes. Such tumors produced a pattern clearly different from that expected with either polyclonal or monoclonal tissues and therefore did not cause incorrect interpretations. In those tumors resistant to Hha I, clonality could usually be assessed by using an additional enzyme, Hpa II. Hpa II, like Hha I, is sensitive to changes in methylation at its recognition site (*10*). As shown in Fig. 1A, there are nine Hpa II sites within the HPRT locus. Hpa II sites 4 to 9 (like Hha I sites H_2 to H_6) are methylated in over 95 percent of active X chromosomes, but at least one of Hpa II sites 4 to 9 is unmethylated in inactive chromosomes (*14, 18*). Hence, an analysis identical to that illustrated in Fig. 1B for Hha I and Bam HI can be performed with the use of Hpa II and Bam HI (*19*). Unambiguous determinations of clonality could be made in 13 of 14 tumors examined by using either Hpa II or Hha I to analyze the HPRT locus; one tumor was resistant to both Hpa II and Hha I digestion. (ii) The strategy outlined here, like other strategies based on X-chromosome inactivation, cannot distinguish between some cases of monoclonal and oligoclonal tumors. In biclonal tumors, for example, 50 percent would appear monoclonal. (iii) Contamination of the tumor with normal tissue due to a large stromal component or to inflammatory cells, can result in the appearance of polyclonality even when the tumor is monoclonal in origin (*2*). Careful histological examination must be done to avoid this potential artifact. However, the analysis of DNA polymorphisms rather than protein polymorphisms offers a significant advantage in this regard. It is virtually impossible to know the relative contribution of tumorous versus normal tissue to the total amount of enzyme in a solid tumor; even a small amount of normal tissue may contribute a relatively large amount of enzyme. However, each normal and tumor cell will contribute the same amount of DNA (in fact, hyperdiploidy in the tumor cells may make the tumor's contribution relatively greater). Hence, simply comparing the number of tumor nuclei with the number of normal nuclei in the sample will provide an upper limit to the amount of DNA contamination by normal cells.

This analysis, based on the HPRT gene, can determine clonality in approximately 25 percent of female tumors. However, since there are many RFLP's in other loci on the X chromosome [for example, see (*20*)] and since changes in methylation often accompany gene activation (*8*), this strategy will probably eventually be applicable to tumors from virtually any female. There are several practical applications for clonal determinations. For example, it has been shown that clonality may in some circumstances be used to distinguish hyperplasia from neoplasia (*21*), and that clonality can detect a preleukemic phase during the remission stage of acute myelocytic leukemia (*22*). The ability to determine clonality in a large number of tumors from female patients should therefore have significant clinical implications.

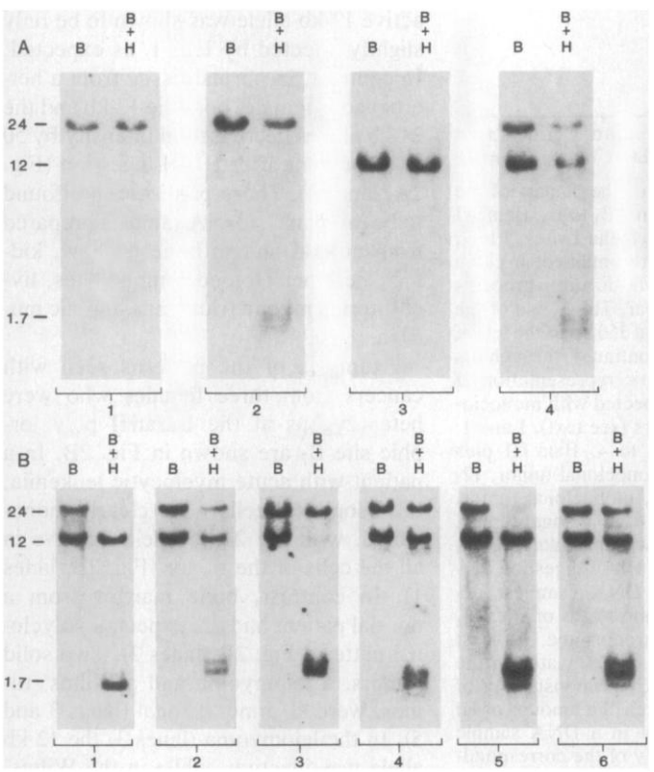

Fig. 2. (A) Results with normal polyclonal tissues. Lanes 1, male with 24-kb allele; lanes 2, female homozygous for the 24-kb allele; lanes 3, male with 12-kb allele; lanes 4, heterozygous female. DNA was prepared from normal colonic mucosa using previously published methods (*24*). DNA was cleaved with Bam HI or Bam HI plus Hha I, using 10 units of each enzyme per microgram of DNA. After electrophoresis through an 0.8 percent agarose gel, DNA was depurinated by soaking the gel in 0.19*N* HCl for 30 minutes [a modification of (*25*)], and then transferred to nitrocellulose by the method of Southern (*26*). Prehybridization washes and hybridization were performed at 60°C in 40 percent formamide, 10 percent dextran sulfate, 0.4 m*M* EDTA, 0.72*M* NaCl, 40 m*M* $NaPO_4$, *p*H 7.0. The probe was the 1.7-kb insert from pPB1.7 (see Fig. 1A) labeled to 1.6×10^8 dpm/μg by nick translation (*27*). Filter washing was carried out at 60°C in the buffers described previously (*28*). Autoradiography was performed for 30 hours at −75°C on Kodak XAR-5 film using Dupont Lightning Plus intensifying screens (*29*). Sizes of DNA fragments in kilobases are given to the right of the figure (determined with lambda phage DNA digested with Hind III). (B) Clonal analysis of human cancers. Lanes 1, acute myelocytic leukemia; lanes 2, normal human marrow; lanes 3, leiomyoma; lanes 4, normal myometrium from the same patient as in lanes 3; lanes 5, Wilms' tumor; lanes 6, normal kidney tissue from the same patient as in lanes 5. DNA was prepared from human cells obtained through bone marrow aspiration (lanes 1 and 2) or surgery (lanes 3 to 6). DNA preparation and hybridization analysis were performed as for (A).

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30 August 1984; accepted 26 November 1984

A Single Recombinant Plasmid Expressing Two Major Outer Surface Proteins of the Lyme Disease Spirochete

Abstract. *A gene bank of DNA from the Lyme disease spirochete was constructed in the plasmid pBR322. Plasmid pTRH32, a recombinant that in* Escherichia coli *expresses the two major outer surface proteins of the Lyme disease spirochete, was identified. One of the recombinant products, designated OspA, represents a surface protein that appears to be common to all Lyme disease spirochetes, whereas the other recombinant product, designated OspB, represents a more variable surface protein. This recombinant plasmid provides a foundation for future studies on the epidemiology and pathogenesis of Lyme disease as well as on the genetic organization of the etiologic agent.*

Lyme disease is a tick-borne disorder characterized by a distinctive skin lesion (erythema chronicum migrans), meningoradiculitis, cardiac abnormalities, and an arthritis (*1*). The etiologic agent of this disorder, a hitherto unknown spirochete (*2*), has been designated *Borrelia burgdorferi* (*3*). This organism has been isolated from the blood, skin, and cerebrospinal fluid of infected patients (*4, 5*), from the tick vectors *Ixodes dammini* (*2, 4*), *I. ricinus* (*6*), and *Amblyomma americanum* (*7*), and from mammals in areas in which the disease is endemic (*8, 9*).

The chronicity of some of the manifestations of Lyme disease, such as the oligoarticular arthritis and meningoradiculitis, suggests that the host cannot effectively rid itself of the infecting agent. Alternatively, the host's immune response to the spirochete may actually induce or accentuate the pathological lesions associated with this disorder. Thus, knowledge of the balance struck between the host's immune system and the spirochete during chronic infection may be a key to understanding the pathogenesis of Lyme disease.

In earlier studies, we identified several antigenic components of the spirochete causing Lyme disease (LD spirochete) by their reactivities with antibodies in the sera of patients with the disease (*10, 11*). One of these components, a surface protein with an apparent molecular weight of 31,000 (31K), was common to all LD spirochetes examined (*12*). More recently, variations were shown to occur in the antigenic determinants and apparent molecular weight of another major outer surface protein (*13*). The apparent molecular weight of this latter protein was approximately 34K (*13*). We now designate the 31K and 34K surface proteins as OspA (outer surface protein A) and OspB, respectively.

Our approach toward understanding the pathogenesis and immunology of Lyme disease and related disorders initially entailed the cloning of LD spirochete DNA and the identification of recombinant clones that express LD spirochete antigens. This approach facilitates the production of individual LD spirochete antigens free of contaminating LD spirochete proteins and animal serum components. We now report the construction of a gene bank of DNA from the LD spirochete in *Escherichia coli* and the isolation of a recombinant clone that expresses two of the major surface antigens of the spirochete.

The LD spirochete strain B31 (A.T.C.C. 35210) was initially isolated from the tick vector *I. dammini* (*2*). This organism was grown in BSK II medium as described (*13*). Chromosomal DNA was obtained (*14*) and partially digested with the restriction enzyme Sau 3AI (Bethesda Research Laboratories); 1.5- to 8-kb (kilobase) fragments were isolated after the DNA was subjected to electrophoresis in low-melting temperature agarose (Seaplaque, FMC Corporation) (*15*). These fragments were ligated into the Bam HI site of dephosphorylated pBR322 (New England Biological Laboratories) with T4 DNA ligase (Bethesda Research Laboratories) by using a 3:1 ratio (by weight) of chromosomal to vector DNA. *Escherichia coli* strain ED8654 (*met gal supE supF hsdR*) was transformed with the ligated mixture as described (*16*). Ampicillin-resistant colonies were obtained and subsequently screened in situ by colony radioimmunoassay (*17*). Our source of antibody reactive with LD spirochete components was synovial fluid from a patient with chronic Lyme arthritis. This synovial fluid has been shown to have a titer of 1:2560 for LD spirochetes in an immunofluorescence assay and to react with several LD spirochete components (*11*). The specificity of this antiserum was enhanced further by adsorption with a sonicate of *E. coli* ED8654 (pBR322) before assay.

Approximately 10,000 colonies were examined by the colony blot assay, and at least three colonies were identified that produced detectable signals in the autoradiographs. Whole cells of these recombinants were lysed with sodium dodecyl sulfate (SDS), and components of the lysates were electrophoretically separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose for Western blot analysis as described (*11, 12*). This analysis confirmed the expression of LD spirochete antigens by recombinants identified in the colony blot assay. One recombinant (pTRH32) was identified that expressed two immunoreactive pro-